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CD44: survival and metastasis in chondrosarcoma

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SUMMARY

Objective: Recent studies have shown abnormal expression of CD44s and some of its isoforms in many human malignancies, but little is known about the presence of CD44 in chondrosarcoma. In this study the expression of CD44s and two variant isoforms was evaluated. It was assumed that abnormalities in these receptor proteins may be associated with clinical outcome of the patients.
Method: Thirty paraffin-embedded chondrosarcoma samples were immunostained with monoclonal antibodies for CD44s, CD44v5 and CD44v6. Two independent examiners who were unaware of the clinical status of the patients evaluated the immunohistochemical results. The percentage of CD44-positive cells was scored semiquantitatively. A rate of higher than 10% was considered as overexpression.
Results: Among the 30 patients (median age 50 years) there were 22 conventional chondrosarcomas, two dedifferentiated chondrosarcomas, two extraskeletal chondrosarcomas, and one periostal, mesenchymal, clear cell and myxoid chondrosarcoma each. In the immunochemistry staining overexpression (>10% of cells) of CD44s was shown in 56.7% (17 of 30), of CD44v5 in 43.3% (13 of 30) and of CD44v6 in 6.7% (two of 30) of the tumors. Four grade III chondrosarcomas (80%) and 10 (71.4%) grade II chondrosarcomas showed overexpression for CD44s, whereas CD44s was overexpressed in only three (27.3%) grade I chondrosarcomas. Cox regression suggests overexpression of CD44s to be an additional prognostic marker for chondroid bone tumors independent of grading and other covariates.
Conclusions: Overexpression of CD44s correlated significantly with metastatic potential and with poorer survival in patients with chondrosarcoma. CD44s might be an independent additional marker, but small sample size remains to be considered.

Introduction

Amongst all malignant bone tumors chondrosarcoma is the second most common, accounting for 10–26% of all osseous malignancies[1–3]. It is the most frequently diagnosed bone tumor in the population older than 50 years[1,3]. The most common skeletal sites affected by chondrosarcoma are the bones of the pelvis followed by the proximal femur, proximal humerus, distal femur and ribs[1]. Clinical symptoms are very unspecific. Radiographic findings often suggest the diagnosis of chondrosarcoma because of identification of typical “ring-and-arc” chondroid matrix mineralization and aggressive features of deep endosteal scalloping and soft tissue extension[4]. Chondrosarcomas range from locally aggressive tumors with almost no metastatic potential to high-grade malignancies with a marked propensity to metastasize[3,5]. Based on nuclear atypia and cellularity chondrosarcomas are classified into three grades[1]. Low-grade chondrosarcomas (grade I) grow slowly and are associated with a very low rate of metastasis and death. On the other hand, high-grade tumors (grade II or III) grow more aggressively and metastasis occurs more frequently, which correlates with poorer survivorship[5,6].

CD44 is a transmembrane glycoprotein belonging to the family of cell adhesion proteins. CD44 is present on the surface of many vertebrate cells, such as chondrocytes, lymphocytes, many types of epithelial cells, fibroblasts, smooth muscle cells and a subset of glial cells in the central nervous system[7].

The human CD44 gene is encoded by a single, highly conserved gene with a length of 50–60 kb and is composed of 20 exons[8,9]. CD44 can be expressed in many isoforms. The five amino-terminal exons and five carboxy-terminal exons code together the standard CD44 protein (CD44s), which has a molecular weight of 85–95 kDa[9]. It is the most broadly expressed CD44 form[10]. By alternative splicing additional variable exons can be inserted into

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the membrane-proximal extracellular domain of the CD44 molecule producing CD44 isoforms[9]. The heterogeneity of the CD44 isoforms is also partially generated by posttranslational, cell-type dependent glycosylation and glycosaminoglycan addition[10].

The most important function attributed to CD44 is to promote cell–cell and cell–matrix attachment, mostly through interactions with hyaluronic acid[11,12]. CD44s has essential functions in lymphocyte adhesion and migration, T-cell differentiation, cell proliferation but also in cell-apoptosis signaling[13,14]. The functions of CD44 variants are less well known. The involvement of CD44v6 in supporting maturation and expansion of hematopoietic progenitor cells was described[15].

Of large importance is the participation of CD44 in tumor growth, invasion and metastasis development. Some CD44 variants in a rat model (homologous to human v6 isoform) are expressed only in cell lines of pancreatic carcinoma and mammary adenocarcinoma that developed metastasis. Moreover, the injection of transfectants that express both CD44 and CD44v6 into non-metastazing tumor cell lines conferred metastatic behavior[16].

Many other studies show the association between extent of CD44 expression with tumor growth and more aggressive behavior in human neoplasms, such as prostate cancer, uterine cervical cancer, gastric carcinoma, breast cancer, lung neoplasms, malignant lymphomas, colon carcinoma, some brain tumors or soft tissue sarcomas[17–28] Overexpression of CD44v5 may play a role in metastatic behavior of some musculoskeletal malignancies, as in osteosarcoma[29,30]. There are reports suggesting that differential CD44v6 expression in chondrosarcoma is associated with malignant transformation[31]. However, in patients with chondrosarcoma, the degree of CD44 expression and its prognostic value have not been reported.

In the present study, the expression profiles of CD44s as well as CD44v5 and CD44v6 isoforms were investigated using immunohistochemical staining in chondrosarcoma specimens from 30 patients. Staining results were correlated with pathological and clinical parameters to estimate its prognostic effect.

Method

Tumor samples

The pathology files of 36 patients with chondrosarcoma who had been diagnosed and treated in the authors' institution were retrospectively explored. Thirty patients whose tissues were available were included into this study. 14 patients were males and 16 were females. The median age was 50 (range, 13–82) years. The median follow-up period was 62.5 (range: 0–233; mean 85.8 ± 19.5) months. The tumors were located in axial skeleton in 18 cases [pelvis (8); vertebra (4); shoulder, (4); thorax (2)] and in the limbs in 10 cases [femur (7); fibula, (1); cuboid (1); and metatarsal (1)]. Two tumors were located extraskeletal. There were 22 patients classified as having conventional chondrosarcomas, two patients as having dedifferentiated chondrosarcomas, two patients as having extraskeletal chondrosarcomas, and each one patient as having periostal, mesenchymal, clear cell and myxoid chondrosarcoma. There were 11 grade I (36.7%), 14 grade II (46.7%) and five grade III chondrosarcomas (16.6%).

The most common treatment was the surgical resection of the tumors in 26 cases. Two patients underwent radiotherapy (one combined with chemotherapy). One patient was treated with radiotherapy after undergoing intralesional resection. Preoperative chemotherapy was performed in two patients and one patient received postoperative chemotherapy. Because of co-morbidity two patients received no therapeutic treatment at all.

R1 was defined as tumor resection margins detectable microscopically, and R2 as macroscopically detectable residual tumor. R0

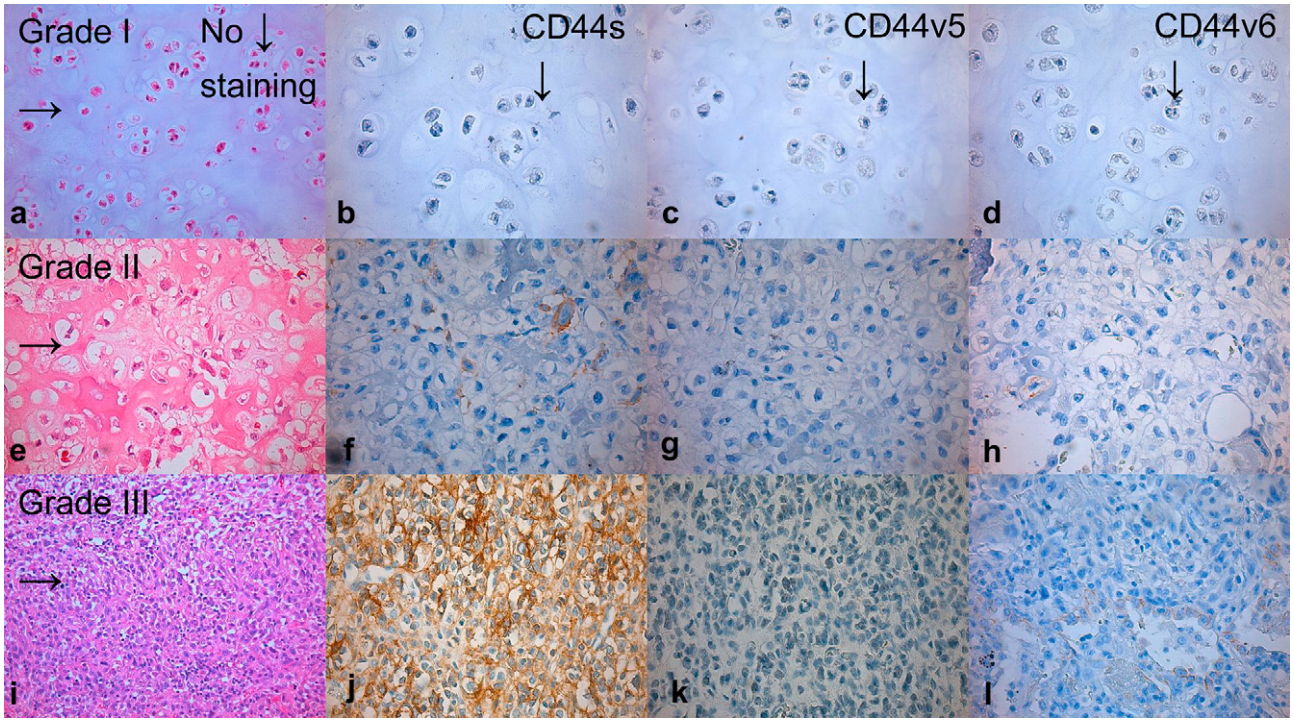

Fig. 1. Grade I chondrosarcomas are composed of moderately cellular mass (a–d). The cells contain small dense nuclei, uniform in size. The stroma is mostly chondroid. Grade II shows increased cellularity (e–h). The nuclei are enlarged, hyperchromatic and atypic. The stroma is frequently myxoid. Grade III tumors (i– l) are more cellular and pleomorphic than Grade II. Mitotic figures are common. The intercellular spaces are filled with myxoid material. The first column shows chondrosarcoma Grades I–III in hematoxilin and eosin (HE) staining. The second column shows CD44s, the third column CD44v5 and the forth column CD44v6. Positive cells are stained brown mostly at the cell membrane. In f and j overexpression with >10% of positive cells is shown. In g, h and l expression of CD44 isoforms is shown, in these examples* with <10% of positive cells.

means that no tumor (macroscopic nor microscopic) occurred on the resection margin. The grading of all tumors was reviewed by the time of the study (RM). Reference pathology from other institutions was obtained. Tumors were categorized as small when they were smaller than 8 cm in diameter. When they were larger than 8 cm in at least one dimension they were considered as large tumors.

Sixteen patients had local recurrences (53.3%). Metastasis developed in 12 patients (40%), including four patients with known metastases at the time of diagnosis. The metastases were treated in three cases with surgery only, in three cases with surgery combined with adjuvant treatments and in one case with chemotherapy only.

Immunohistochemical staining and staining analysis

The selected, paraffin-embedded tissues were immunostained with mouse monoclonal antibodies against CD44s (Clone: SFF-2, dilution 1:10 000), CD44v5 (Clone: VFF-8, dilution 1:500) and CD44v6 (Clone: VFF-18, dilution 1:8000), all from Bender MedSystems. The sections were dewaxed by xylene rinsing and rehydrated gradually through graded alcohols. The slides were rinsed with distilled water and placed in a jar with a 1:10 diluted Dako Target Retrieval Solution buffer (S 1699) at a pH of 6.1 (Dako). The jar was heated in a steamer for 30 min. After cooling and rinsing with distilled water and Dako S 3006 buffer, the slides were placed in a Dako Autostainer plus immunostaining system. Immunostaining was performed using a labeled streptavidin–biotin method (Dako REAL Detection System Peroxidase/DAB+), the staining reaction being based on 3,3'-diaminobenzidine (DAB). The stained slides were rinsed with distilled water and stained for 5 min with hemalaun as counterstain. Finally, the sections were rinsed with water and treated with graduated-density alcohol and with xylol. As positive controls skin tissues were used.

The immunohistochemical results were evaluated by two independent investigators who were blinded to the clinical information. The mean values of their results were used as basis for following calculations. The Cornbach alpha averaged 0.979 for CD44s, 0.946 for CD44v5 and 0.751 for CD44v6 indicating a high interobserver reliability. The percentage of CD44-positive cells was scored semiquantitatively as: negative when there were less than 10% of positive cells, and overexpression when there were more than 10% of positive cells according to Endo *et al.*[32].

Statistical analysis

The data were analyzed with SPSS analytic software. Kaplan–Meier method was used to estimate the survival. Survival functions were compared by using the Logrank test. To determine whether CD44 stainings are new independent prognostic variables, a multivariate analysis with grading, resection margins, sex, age, tumor size and location (trunk vs extremities) as covariates, a Cox regression (Wald test) was performed. Results were expressed as hazard ratios with confidence intervals. High hazard ratios reflect a high impact of the parameter. For all tests, $P < 0.05$ was considered to be statistically significant.

Results

CD44s was expressed in 73.3%, CD44v5 in 80.0% and CD44v6 in 33.3% of the tumors (Fig. 1) including also specimen with <10% positive cells. 56.7% of the chondrosarcoma showed overexpression of CD44s with >10% positive cells. Overexpression of CD44v5 was shown in 43.3% of the cases and overexpression of CD44v6 was

Table I
Patient characteristics

No.	Sex	Age at diagnosis	Histology of chondrosarcoma	Grading	Localization	Therapy	Resection margins	Recurrence	Metastasis	Metastasis free survival time	Death	Survival time/observance time (months)
1	Female	55	Conventional	Grade I	Femur	Surgery	R0	Yes	Yes	42	Yes	55
2	Male	27	Conventional	Grade I	Pelvis	Surgery	R2		Yes	0	Yes	6
3	Male	67	Conventional	Grade I	Pelvis	None	No Op				Yes	12
4	Male	75	Conventional	Grade II	Pelvis	Surgery	R0	Yes	Yes	63	Yes	74
5	Male	33	Conventional	Grade II	Fibula	Surgery	R0	Yes				85
6	Female	26	Conventional	Grade I	Spine	Surgery	R0	Yes				233
7	Female	50	Conventional	Grade III	Shoulder	Surgery + chemotherapy	R0	Yes	Yes	0	Yes	28
8	Male	75	Conventional	Grade II	Spine	Radiotherapy + chemotherapy	No Op					83
9	Male	36	Conventional	Grade I	Spine	Surgery	R0	Yes				101
10	Female	37	Conventional	Grade I	Pelvis	Surgery	R0	Yes				161
11	Male	66	Dedifferentiated	Grade III	Femur	Surgery	R0	Yes	Yes	2	Yes	5
12	Female	72	Conventional	Grade II	Pelvis	Surgery	R1					0
13	Male	52	Conventional	Grade II	Shoulder	Surgery	R1	Yes	Yes	78		186
14	Female	82	Conventional	Grade I	Pelvis	Radiotherapy	No Op	Yes				43
15	Male	37	Conventional	Grade II	Pelvis	Surgery	R0	Yes	Yes	2	Yes	179
16	Female	62	Clear-cell	Grade II	Femur	Surgery	R2				Yes	97
17	Female	50	Conventional	Grade II	Foot	Surgery	R0	Yes	Yes	23		25
18	Female	15	Conventional	Grade I	Shoulder	Surgery	R0	Yes				182
19	Female	72	Dedifferentiated	Grade III	Femur	None	No Op		Yes	0	Yes	4
20	Male	38	Conventional	Grade II	Foot	Surgery	R0					129
21	Male	36	Conventional	Grade I	Spine	Surgery	R0	Yes	Yes	66	Yes	66
22	Female	61	Extraskeletal	Grade II	Extraskeletal	Surgery + chemotherapy	R0					217
23	Male	74	Extraskeletal	Grade III	Extraskeletal	Surgery	R0	Yes	Yes	39	Yes	57
24	Male	45	Conventional	Grade I	Shoulder	Surgery	R0					214
25	Female	44	Conventional	Grade II	Thorax	Surgery	R0					38
26	Female	33	Periosteal	Grade II	Thorax	Surgery	R0	Yes				59
27	Female	37	Conventional	Grade I	Femur	Surgery	R0					205
28	Female	51	Conventional	Grade II	Femur	Surgery	R0					1
29	Male	82	Myxoid	Grade II	Femur	Surgery + radiotherapy	R2				Yes	2
30	Female	13	Mesenchymal	Grade III	Pelvis	Surgery + chemotherapy	R0		Yes	0		27

shown in 6.7% cases. CD44 staining was primarily observed over the cell membrane of tumor cells. The CD44 expression was not age or sex dependent (Table I).

Association of overexpression of CD44 isoforms with clinicopathologic characteristics

Kaplan–Meier analysis showed that overexpression of CD44s was associated with decreased metastatic free survival time of chondrosarcoma ($P < 0.01$, Fig. 2) In addition, in survival analysis patients with chondrosarcoma that expressed CD44s in >10% of the cells had a significantly shorter survival time (average: 110 months vs 194 months; $P < 0.01$, Fig. 3).

Cox regression revealed that the variable overexpression CD44s was independent from the variable grading and all other applied covariates for survival (hazard ratio 46.70 and $P = 0.0074$, Table II) and metastasis (hazard ratio = 78.87, $P = 0.021$, Table III).

Overexpression of CD44v5 was not associated with either the appearance of metastasis ($P = 0.77$) and poorer survival in patients ($P = 0.55$) with chondrosarcoma in the Kaplan–Meier analysis (Figs. 2 and 3). In Cox regression, overexpression of CD44v5 was not found to be an independent prognostic variable for survival (hazard ratio = 10.48, $P = 0.14$, Table II). Cox regression showed, however, that it may be an independent prognostic marker for metastasis (hazard ratio = 100.82, $P = 0.045$, Table III). Thus, the statistical findings for CD44v5 are inconsistent.

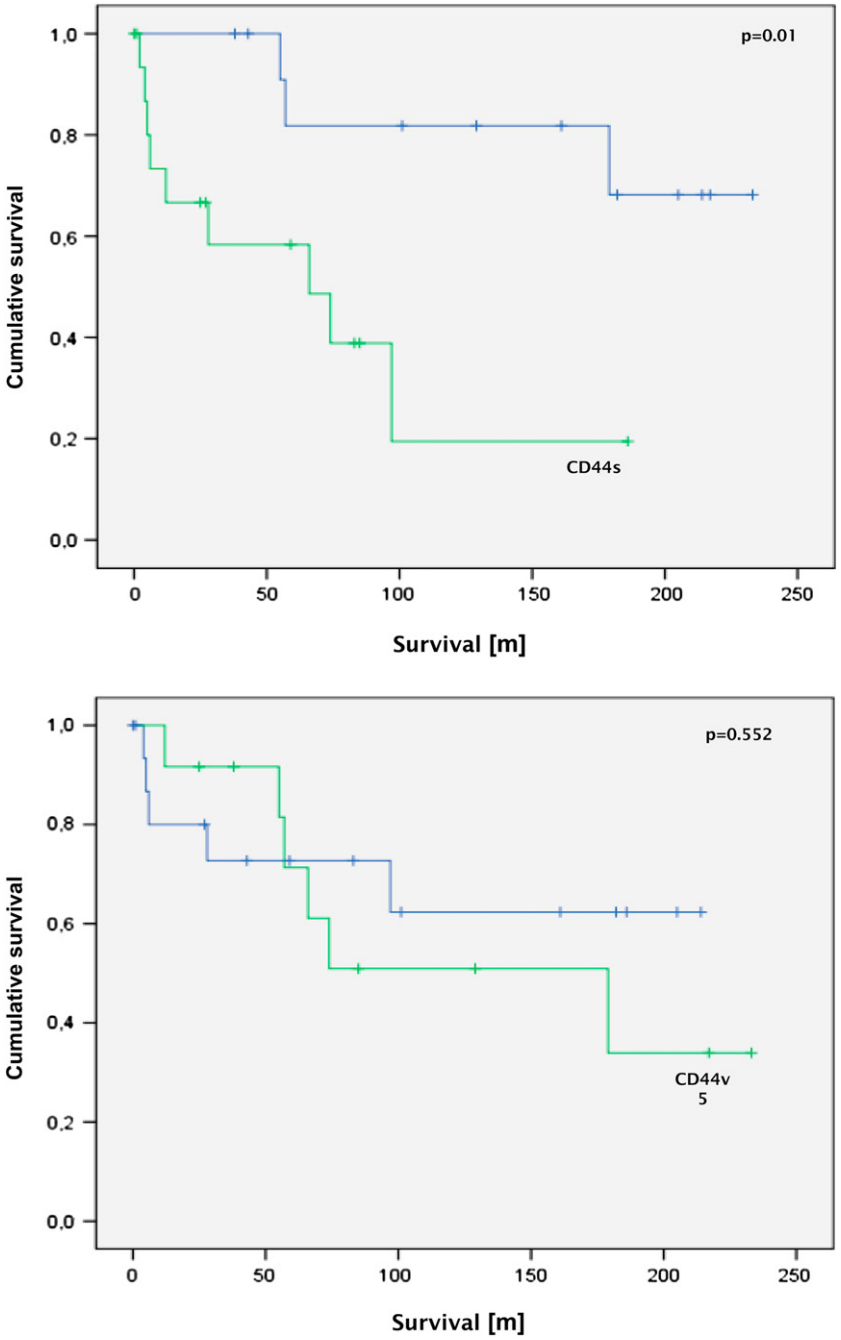

Fig. 2. Cumulative metastasis free survival (Kaplan–Meier) in dependence of expression of CD44s and CD44v5: blue graphs indicate CD44 negative patients. Green graphs outline the survival for patients positive for the respective CD44 isoform.

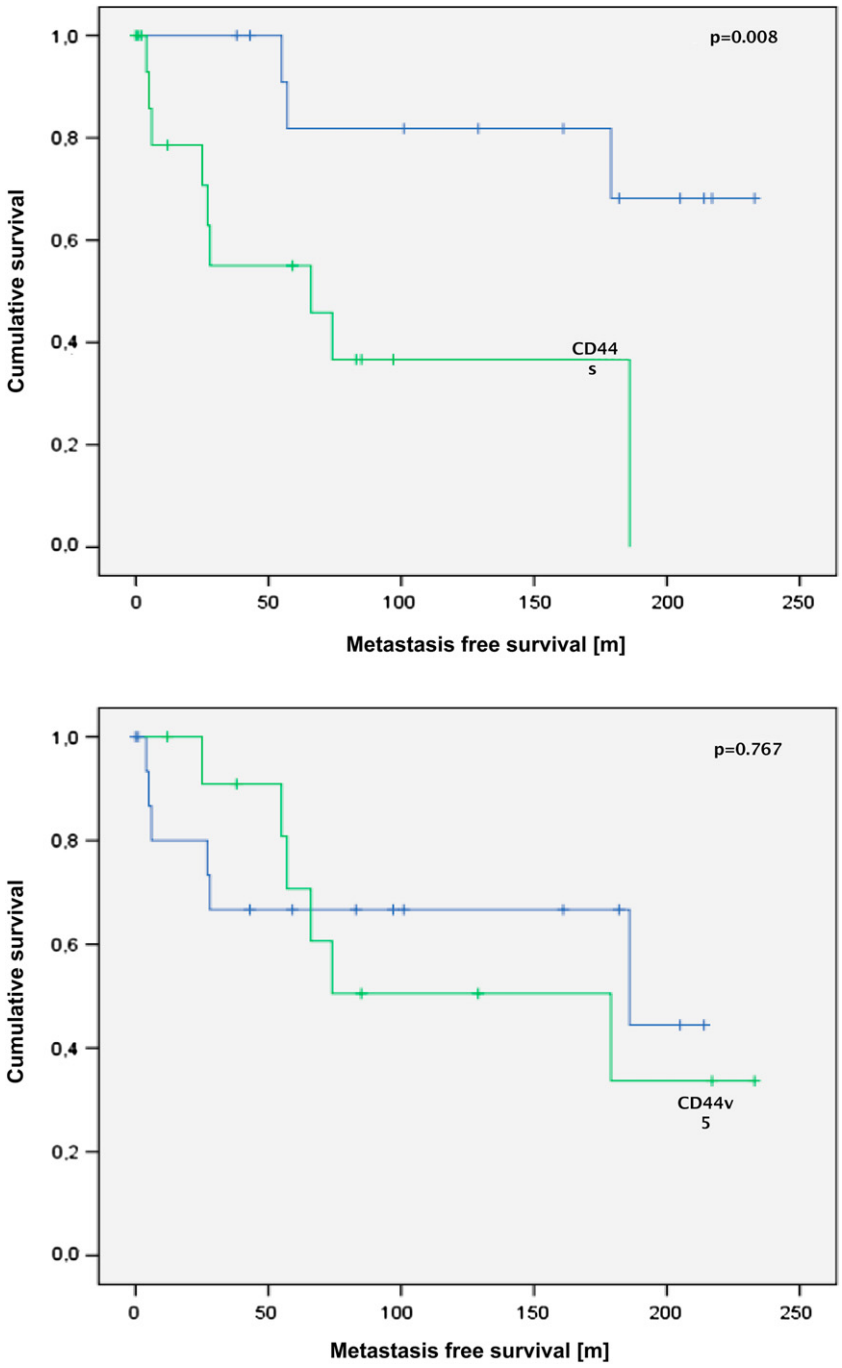

Fig. 3. Cumulative survival in dependence of expression of CD44s and CD44v5 (Kaplan–Meier): blue graphs indicate CD44 negative patients. Green graphs outline the survival for patients positive for the respective CD44 isoform.

Only two cases showed overexpression of CD44v6 and thus a Kaplan–Meier analysis was not performed for this subgroup. In the Cox regression, CD44v6 was not found to be an independent variable for neither survival (hazard ratio = 0.24, $P = 0.42$) nor metastasis (hazard ratio = 0.71, $P = 0.83$).

Discussion

There are many studies describing the presence of CD44 and its isoforms (especially CD44v5 and CD44v6) in normal and degenerative cartilage cells[33–36]. In cartilage, the hyaluronic acid receptor plays an important role in cell attachment to the extracellular matrix. Moreover, CD44 affects the metabolism and proliferation of cartilage cells[37,38]. Other studies characterize the role of CD44 in tumor growth and metastasis development in cartilage. Ishida *et al.* showed that CD44 may play an important role in normal and abnormal functions in cartilaginous cells through its adhesion to hyaluronic acids. In their study, chondrosarcoma-derived chondrocyte-like cell-line HCS-2/8 effectively adhered to hyaluronic acids through cell surface CD44. The adhesion was also involved in cellular signaling, which induced proliferation and expression of c-myc mRNA as well as TGF-beta mRNA expression within the cells[39]. On the other hand treatment with CD44 monoclonal antibodies in human chondrosarcoma cell lines reduced the cell

Table II
Results of a Cox regression for survival considering overexpression of CD44 isoforms, grading and risk factors: results were expressed as hazard ratios with confidence intervals and *P*-values from Wald test (*likelihood-ratio test). High hazard ratios reflect an increased risk

Death	Hazard ratios	95% Confidence interval	*P*-values
CD44s positive	46.70	2.81–777.64	0.0074
CD44v5 pos.	10.48	0.48–230.80	0.14
CD44v6 pos.	0.24	0.008–7.35	0.42
Grading GII vs GI	0.004	0.00002–0.86	0.044
Grading GIII vs GI	15.24	0.29–792.62	0.18
R0	0.42	0.009–19.28	0.65
R1 and R2	8.49	0.14–521.26	0.31
Female sex	1.29	0.29–55.94	0.32
Age at diagnosis	1.07	0.95–1.20	0.26
Location at extremity vs trunk	0.92	0.04–21.19	0.96
Tumor size > 8 cm	10.38	0.57–188.16	0.11

viability by induction of chromatin condensation, nuclear fragmentation and apoptotic body formation[40]. Another study showed, that CD44 stimulation facilitates invasion of chondrosarcoma cells[41]. Cartilaginous CD44 takes active part in tumor origin and dissemination but to date, there is little known about the correlation between the CD44 expression and clinical outcome of patients with chondrosarcoma. The most valuable predictor of patients' outcome at this time is the histological grade[6,42].

To our knowledge this study is the first to investigate the expression of CD44 in chondrosarcomas in correlation with clinical outcome. The most important finding of the current study is the prognostic significance of the expression of CD44s in malignant cartilaginous tumors. Cox regression revealed that CD44s is a significant variable independent from grading with respect to survival and metastasis. Cox regression showed that overexpression of CD44v5 may be an independent prognostic marker for metastasis but not for survival. This was not supported by results of the Kaplan–Meier analysis for CD44v5 and statistical tests remain inconsistent concerning this subtype.

CD44s and maybe also CD44v5 could be useful as prognosis predictor in malignant cartilaginous tumors. However, there are some limitations to these statements due to sample size. The results have to be handled with care. Early excitement over the prognostic value of CD44 isoforms in breast cancer turned out to be substantially less useful when larger studies were performed[43,44]. Another limitation to the study is that grading of chondrosarcoma remains somewhat subjective in dependence of the examining pathologist. Biopsies from large tumors might be prone to sampling bias. Some of the histologically diagnosed G1 tumors that developed metastasis might eventually have been or progressed to higher grade tumors.

This analysis contains all histological subtypes of chondrosarcoma. They have in common that they are malignancies producing chondroid matrix, which justifies putting them together. Limiting the analysis to conventional chondrosarcomas would still come with a large degree of heterogeneity among the cases, since already these are morphologically and clinically very diverse.

Table III
Results of a Cox regression for metastasis analog to Table II

Metastasis	Hazard ratios	95% Confidence interval	*P*-values
CD44s positive	78.87	1.92–3242.97	0.021
CD44v5 pos.	100.82	1.11–9179.81	0.045
CD44v6 pos.	0.71	0.03–18.01	0.83
Grading G2 vs G1	0.05	0.002–1.21	0.065
Grading G3 vs G1	267.25	5.10–13993.19	0.0057
R0	0.54	0.008–34.32	0.77
R1 and R2	12.03	0.20–723.04	0.23
Female sex	7.65	0.61–95.65	0.11
Age at diagnosis	0.98	0.92–1.04	0.43
Location at extremity vs trunk	2.15	0.19–24.24	0.53
Tumor size > 8 cm	45.34	1.23–1673.45	0.038

These data support the thesis that CD44s expression is related to metastatic potential and patients' outcome in some tumors. Bhatavdekar *et al.* showed in a multivariate analysis that CD44 overexpression was the most important indicator of an unfavorable prognosis for overall survival in patients with colorectal carcinoma[45]. Strong CD44 expression was associated with shorter 10-year survival rate among patients with gastrointestinal lymphoma[22]. Also in bladder cancer association between strong expression of CD44s and unfavorable outcome was found. Moreover, the expression intensity of CD44s was significantly related to pathological grading of bladder cancer[46]. However, increased levels of CD44 expression not necessarily correlates with poor prognosis in every malignancy. In some tumors, cases with low CD44 expression demonstrate higher metastatic potential than cases with high CD44 expression[47,48].

Also CD44v6 seems to be important for malignancies to generate more aggressive growth and potential for metastasis. CD44 variants containing exon v6 sequences appeared mostly at the advanced stage of tumor development and were more prevalent in metastatic colorectal carcinomas[28]. Elevated levels of CD44v6 expression were observed in disseminated large-cell lymphomas[49]. Patients with primary breast cancer who expressed CD44 with v6 epitope had a poor overall survival compared to patients with v6 negative tumors[43]. Bosch *et al.* postulated that CD44v6 may play a role in origin of abnormal cartilage phenotype. In their study the presence of fibrocartilaginous neoplastic masses was associated with lack of the CD44 variant containing v6[31]. However, only three low-grade chondrosarcomas were included in their work and no correlation between patients' prognosis and CD44 staining was provided. In the presented data, there were only little numbers of tumors and cells staining positive for CD44v6 and only two tumors met the criteria for overexpression. Thus, no significant findings for overexpression of v6 were found.

As these data demonstrated, CD44 may play a crucial role in growth and dissemination in many human tumors. It is hypothesized that the expression of CD44 and CD44v5 could contribute to invasive growing and developing of metastasis in chondrosarcoma, too. We assume that a simple immunohistochemical staining of chondrosarcoma samples with CD44s and CD44v5 could help with grading of chondrosarcoma and prognosis of disease. Power of this study is limited by sample size and the heterogeneity of appearance of chondrosarcoma. This might also affect the results of the multivariate analysis performed and might explain why some of the known negative predictors for survival and metastasis in chondrosarcoma failed statistical significance in the presented setting. However, Cox regression and Kaplan–Meier analysis suggested that CD44s may be a prognostic variable independent from grading and the other included covariates with respect to survival and metastasis.

Conclusion

In conclusion, positive cell staining for CD44s was significantly associated with the metastatic potential and survival in patients with chondrosarcoma. The results of this study demonstrate that CD44s could be an additional independent prognostic marker for patients with chondrosarcoma.

Conflict of interest

All authors declare to have nothing to disclose and to have no potential conflict of interest that could inappropriately influence the presented work.

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